PHYTOCHEMICAL AND MOLECULAR ANALYSIS OF SOME MEDICINAL PLANTS OF *LABIATAE* FAMILY GROWING AT DIFFERENT ALTITUDES ON SAINT KATHERINE MOUNTAIN, SOUTH SINAI, EGYPT

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Medicinal plants are used by 80% of the world population for their basic health needs. Traditional systems of medicines are prepared from a single plant or combinations of more than one plant. These efficacies depend upon the current knowledge about biological property of medicinal plants which in turn depends upon the occurrence of primary and secondary metabolites (Vinoth *et al.*, 2011).

Genus *Nepeta*, *Ballota* and *Teucurum* remain quite important medicinal plants belonging to family of *Labiatae* that contains a wide variety of chemicals and volatile oils which are common to many members of this family. It was found to contain variety of diterpenoids, iridiods, phenolic compounds and flavonoids **(**Naghibi *et al*., 2005)**.** *Nepeta* is a genus of about 250 species and only one species of this genus was recorded in Egypt and this species (*Nepeta septemcrenata*) is endemic for Sinai (Tackholm, 1974). It was reported that *Nepeta* plants were prepared as tea and used in traditional medicine as anthelmintics, febrifuges, expectorants, to treat bronchitis, bites, stings of and scorpions (Arnold *et al*., 1993). *Ballota undulata* plant is used by Bedouins as a remedy for the treatment of wounds, scorpion, bee, and wasp stings (Sathiyamoorthy *et al.*, 1997). The aqueous extract of *B. undulata* was tested for antitumor and antimalarial activities (Sathiyamoorthy *et al*., 1999). *Teucrium polium* is a perennial herb having a pleasant aromatic odor and a bitter taste. This species is used in treatment of digestive and respiratory disorders, abscesses, gout and conjunctivitis, in the stimulation of fat and cellulite decomposition. It possesses anti-inflammatory, antioxidative, antimicrobial, ant diabetic and antihelmintic effects (Darabpour *et al*., 2010).

Plant secondary metabolites are organic molecules that are not involved in the normal growth and development, but often playing an important role in plant response to both of abiotic and biotic stresses. It is include the phenolic acids, flavonoids, alkaloids, steroids, terpenoids,

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carotenoids, lignans, tannins, cardiac glycosides, and many others (Nonita and Mylene, 2010). These compounds constitute the bioactive compound in several medicinal and aromatic plants. All secondary metabolites have specific function as like saponins have antifungal activity **(**Sodipo *et al*., 1991**)**, some alkaloid may be useful against HIV infection **(**McMahon *et al*., 1995**)**, flavonoids have strong anticancer activity (Noble, 1990) and tannin have antimicrobial activity.

Phytochemical screening is one of the techniques to identify and characterize new sources of therapeutically important compounds like alkaloids, flavonoids, phenolics, steroids, tannins, saponins etc. present in the plant extracts. Knowing the chemical constituents of plants is necessary because such information will be of value for the synthesis of new bioactive compounds for treating the specific disease. Numbers of plants were screened for secondary metabolites for their medicinal values in *Cichorium intybus*, *Eclipta alba*, *Morinda citrifolia*, *Mangifera indica*, *Cissus populnea* and *Bauhinia tomentosa* **(**Boopathi and Sivakumar, 2011; Soladoye and Chukwuma, 2012).

Number of environmental factors such as climate, altitude, rainfall and other conditions may affect the quality of herbal ingredients present in a particular species which in turn may produce major variations in the bioactive compounds present in the plants (Kokate *et al*., 2004**)**. In mountainous environment, variation in altitude offers wide variety of environmental conditions. In general, with increase in elevation, stressors such as temperature, pressure, light intensity, rainfall, and partial pressure of metabolic gases are known to influence plant secondary metabolism (Purohit, 1998). Qualitative and quantitative phytochemical screening of those secondary metabolites will help to understand a variety of chemical compounds produced by plants and to extract, purify and identify the bioactive compounds.

Genetic variation between plants can be a major source of variation in plant secondary metabolites, and can considerably affect the amounts and type of metabolites produced within a single plant species (Poulev *et al*., 2003). Genetic diversity of species is related to geographic distribution, mode of reproduction, breeding system, and seed dispersal mechanism. Therefore, successful management and preservation of populations of rare, threatened, or endangered species depend on a complete understanding of the species, including levels and structure of genetic variation **(**Arzate-Fernández *et al*., 2005**)**. Genetic diversity has been traditionally assessed by morphological markers. However, these markers are time consuming and often the testing procedures are complex or unreliable. Molecular markers offer fast screening and a wide range of novel approaches to improve the selection strategies in plant breeding (Ibitoye and Akin-Idowu, 2010). Polymerase chain reaction (PCR) molecular markers like random amplified polymorphic DNA (RAPD) and inter-simple sequence repeats

(ISSRs) would be an option because of the lower level of skill required, low cost per assay, and the ready availability of primers allow the scanning of the entire genome and efficient genotype characterization. Thus, because of their characteristics and efficiency for detecting polymorphisms, the RAPD and ISSR markers have been successfully used to calculate the intra or inter-specific genetic diversity in different wild species (Li *et al*., 2011).

The objectives of this study were to (1) analysis the genetic polymorphism of *N. septemcrenata*, *B. undulata* and *T. polium* plant species growing at three different altitudes of Saint Katherine Mountain, South Sinai, Egypt by using RAPD and ISSR markers (2) analysis the qualitative and quantitative phytochemical of three species in related to three different altitudes and (3) determine if the genetic polymorphism, the qualitative and quantitative phytochemical among the species are related to an altitudinal gradient.

MATERIALS AND METHODS

A. Collection and Identification of Plant samples

Three plant species belong to family *Labiatae* (*Nepeta septemcrenata*, *Ballota undulata* and *Teucrium polium*) were used in this study. Selection of these three species depended on that these species can be collected from all altitudes profiles. Aerial parts of at least five individuals from each species were collected on August, 2013 from three different elevation ranks (1800 m above sea level [a.s.l.], 2200 m a.s.l and 2600 m a.s.l.) on Mountain of Saint Katherine, South Sinai, Egypt. The coordination points for plant sample sites are indicated in Table (1) and Fig. (1). The plants were kindly identified with Dr. Mohamed Metwaly Sayed Moursy, lecturer of Plant Ecology, Department of Botany, Faculty of Science, Al-Azhar University. A voucher specimen has been deposited in the Herbarium of Department of Botany and Microbiology, Faculty of Science, Al-Azhar University, Naser City, Cairo, Egypt.

B. Methods

1. Phytochemical analysis

1.1. Methanolic extract preparation

About 5 g of air-dried plant powder were reflaxed with 2.5 L of 70% methyl alcohol for 6 hours, and then filtered. The residue powder was then washed several times with hot alcohol. The combined filtrates were concentrated under reduced pressure at 50°C, and then used for the following tests:

1.2. Phytochemical qualitative screening

Preliminary phytochemical screening including steam distillation of volatile oils (Balbaa *et al*., 1981), test for Alkaloids (Woo *et al*., 1977), test for glycosides (Treare and Evan, 1985), test for cardiac glycosides (Treare and Evan, 1985**)**, test for saponins (Kokate and Klinger-Hand, 2001), test for phenols (Ahmad *et al*., 2005), test for phytosterols (Brieskorn *et al*., 1961), test for tannins

(Treare and Evan, 1985), test for flavonoids (Khandeal, 2008).

1.3. Phytochemical quantitative screening

1.3.1. Estimation of Total Flavonoid Content (TFC)

The amount of total flavonoid content in extract was determined by aluminum chloride assay through Colorimetric method **(**Samatha *et al*., 2012**)**. A 0.5ml aliquot of appropriately diluted sample solution was mixed with 2 ml of distilled water and subsequently with 0.15 ml of a 5% $NaNO_2$ solution. After 6 minutes, 0.15 ml of a 10% $AlCl_3$ solution was added and allowed to stand for 6 minutes, then 2 ml of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final volume to 5 ml, then the mixture was thoroughly mixed and allowed to stand for another 15 minutes. Absorbance of the mixture was determined at 510 nm versus prepared water blank. Rutin was used as standard compound for the quantification of total Flavonoid. Total flavonoid content was expressed as mg rutin/g dry weight (mg rutin/g DW), through the calibration curve of Rutin. All samples were analyzed in three replications.

1.3.2. Estimation of total phenolic content (TPC)

The amount of total phenolic in extract was determined with the Folin Ciocalteu reagent **(**Maurya and Sing, 2010**)**. Gallic acid was used as a standard and the total phenolic was expressed as µg/mg gallic acid equivalent (GAE). All samples were analyzed in triplicate. The Folin Ciocalteu reagent is sensitive to reducing compounds including polyphenols, thus, they produce a blue color upon reaction. This blue color was measured at 760 nm spectrophotmetrically**.** Line of Regression from Gallic acid was used for estimation of unknown phenol content.

1.3.3. Estimation of total saponins

Two grams of plant parts were dispersed in 20 ml of 20% ethanol. The suspension was heated over a hot water bath for 4 hours with continuous stirring at about 55°C. The mixture was filtered and the residue was re-extracted with another 200 ml of 20% ethanol. The combined extracts were reduced to 40 ml over water bath at about 90°C. The concentrate was transferred into a 250 ml separating funnel and 20 ml of diethyl ether was added and shaken vigorous. The aqueous layer was recovered while the ether layer was discarded. The purification process was repeated and 60 ml of n-butanol was added. The combined n-butanol extract were washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated in a water bath. After evaporation, the samples were dried in the oven to a constant weight. The saponins content was calculated in percentage according to Obadoni and Ochuko (2001).

1.3.4. Estimation of total alkaloids (Gravimetric Method)

About (2 g) of the plant parts were extracted with 90% ethanol till exhaustion

(tested with Mayer's reagent). The alcoholic extract of the plant was concentrated under reduced pressure until dryness at a temperature not exceeding 40°C, acidified with HCl (3%), and filtered; the filtrate obtained was extracted with chloroform to remove acid alkaloid portion. The acidic aqueous layer was adjusted to alkaline media with ammonia and the liberated alkaloid base portion was extracted with chloroform till exhaustion (tested by Mayer and Dragendorrf's reagents). The chloroform extract was filtered over anhydrous sodium sulfate and evaporated under reduced pressure till dryness, then weighed it to calculate the percent w/w **(**Woo *et al*., 1977).

1.3.5. Estimation of total tannins (Gravimetric Method)

This method depends on quantitative precipitation of tannin with copper acetate solution, igniting the copper tannate to copper oxide and weighing the residual copper oxide **(**Ali *et al*., 1991). Two grams of aerial parts were separately extracted for about one hour with two successive quantities, each of 100ml of acetone-water (1:1) and then filtered. The combined extract, in each case, was separately transferred into a 250 ml volumetric flask and adjusted for volume with distilled water. Each extract was quantitatively transferred to a 500 ml beaker and heated till boiling, then 30 ml of 15% aqueous solution of copper acetate was added with stirring. The precipitate of copper tannate was collected on ashless filter paper and the precipitate was ignited in a porcelain crucible (the crucibles were previously ignited to a constant weight at the same temperature). Few drops of nitric acid were added to the residue and reignited to constant weight. The weight of copper oxide was determined and the percentage of tannin was calculated according to the following correlation: Each 1 g of Cuo = 1.305 g tannins.

1.4. Molecular analysis

1.4.1. DNA isolation

The genomic DNA was extracted from approximately 100 mg air-dried plant. The extraction procedure was the cetyltrimethylammonium bromide method that was reported by Arzate-Fernández *et al.* (2005). Five DNA samples from each species through each altitude were dissolved together as bulk DNA.

1.4.2. DNA quantification

Quality of the extracted DNA was checked by running on 0.8% agarose gel. Purity and concentration of genomic DNA was estimated by calculating the ratio of optical densities measured at 260-280 nm with a spectrophotometer (Thermo Scientific Type UV1, England). Appropriate dilutions of DNA were made for further amplification and PCR analysis.

1.4.3. Primers

A set of twenty 10-mer oligonucleotides was analyzed for RAPD-PCR and a total of sixteen primers were tested for ISSR. Based on the accurate amplified

bands profiles and the produced polymorphic patterns of DNA fingerprinting selected five different primers were chosen for RAPD-PCR and another five primers for ISSR (Table 2). The remaining primers were not considered for compiling the results because they were either not polymorphic or did not give clear amplifications.

1.4.4. PCR reactions

The amplification reactions were carried out in 25µl per tube, containing 2µl DNA (20 ng), 1 unit of Taq DNA polymerase enzyme, 2 µl 10X buffer, 2 µl $MgCl_2$ (25 mM), 2 µl dNTPs (2.5 mM of each), 2 µl primer (10 pmol) and 14.8 µl H_2O. Both RAPD and ISSR amplification reactions were carried out on a Perkin-Elmer Gene Amp PCR system (model 2400), and each reaction was repeated twice.

1.4.5. PCR conditions

The RAPD amplifications occurred under the following conditions: an initial denaturation step at 94°C for 7 min and 30 cycles at 94°C for 1 min, 35°C for 1 min and 72°C for 2 min; the final elongation step was at 72°C for 6 min. The following conditions were used for ISSR amplifications: an initial denaturation step of 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, a primer annealing step at 52°C for 45 s, and an extension at 72°C for 2 min; then a final extension was carried out at 72°C for 5 min. The annealing temperature varied according to the melting temperature of each primer.

1.4.6. Band analysis

The reaction products were analyzed by electrophoresis on 1.4% agarose gels, stained with ethidium bromide and photographed under UV transilluminator by digital camera with UV filter adaptor. The synthetic DNA, ladder 100 bp (Pharmacia) was employed as molecular markers for bands molecular weight. Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Profiles were considered different when at least one polymorphic band was identified. Fragments were scored as 1 if present or 0 if absent based on standard marker using GelAnalyzer 3 (Egygene) software.

1.5. Statistical analysis

All the analyses were performed in triplicate and the results were statistically analyzed and expressed as mean (n = 3) ± standard error of means. Pairwise combinations, genetic similarity and genetic distances were estimated following Lynch (1990 and 1991). The computer package SPSS was used to construct a dendrogram based on the matrix of distance using Unweighted Pair Group Method with Arithmetic averages (UPGMA) (Sneath and Sokal, 1973).

RESULTS AND DISCUSSION

1. Qualitative and quantitative phytochemical analysis

Elevations strongly influence landscape topography, geology, rainfall amount and consequently soil moisture

and texture, ground-water depth, hydrology, evaporation, soil type and vegetation itself (Knoop and Walker, 1985). The influence of altitude, as an ecogeographical factor, on the kind and quantity of secondary metabolites extracted from three plant species i.e. *N. septemcrenata*, *B. undulata* and *T. polium* collected from Saint Katherine Mountain has been investigated. Qualitative screening revealed presence of alkaloids, glycosides, cardiac glycosides, saponins, phenolic, tannins and flavonoids in methanol extracts for all three studied species. Sterols, diterpens and volatile oils were absent from this extract (Table 3). The current results were in agreement with previous phytochemical screening of *N. septemcrenata* herb that revealed presence of phenolic and terpenoid compounds (Balbaa *et al.*, 1981). Also, our results were compatible with the phytochemical study of *Ballota* genus in which various polyphenols including phenylpropanoid derivatives, flavonoids and phenolic acids have been isolated and identified (Citoglu *et al.*, 2005).

It was observed during the present study that the quantity of phytochemicals, especially both of total flavonoids and total phenolic acids were greatly affected in response to the difference in elevation ranks. A great diversity was recorded as regards these two secondary metabolites constituents of the plants among the different elevation ranks with the three species under investigation (Table 4).

Our results reported that methanolic extract from *N. septemcrenata* species have the maximal values (201 mg/g dry wt. and 270 mg/g dry wt.) from total flavonoids and total phenolic acids, respectively at higher elevation (2600 m) and minimal values (145 mg/g dry wt. and 235 mg/g dry wt.) at lower elevation (1800 m) (Table 4). Methanolic extract from *B. undulata* have the maximal values (208 mg/g dry wt. and 267 mg/g dry wt.) from total flavonoids and total phenolic acids, respectively at higher elevation (2600 m) and minimal values (168 mg /g dry wt. and 211 mg/g dry wt.) at lower elevation (1800 m) (Table 4). Methanolic extract from *T. polium* have the maximal values (257 mg/g dry wt. and 299 mg/g dry wt.) from total flavonoids and total phenolic acids, respectively at higher elevation (2600 m) and minimal values (195 mg/g dry wt. and 254 mg/g dry wt.) at lower elevation (1800 m) (Table 4). It could be noted that the curve showing of changes in total flavonoids and total phenolic acids showed similar trends in the different species. The high production of these phytochemicals may be attributed to fluctuation in temperature and non-availability of nutrients. Moreover, this increase in the flavonoid and phenolic contents with increase in altitude may be ascribed as a response of plants to enhanced UV-B radiation and decreased temperatures which elicit amplified biosynthesis of UV-absorbing and antioxidant phenolics in plant (Spitaler *et al.*, 2008).

The concentration and composition of phenolic compounds varies extensively both within and among species. Large

differences in both ambient concentrations and the responsiveness of phenolics to environmental manipulations have been observed in plants of the same species but from different arctic areas (Graglia *et al*., 2001). Such data suggest that the effect of environmental changes on plant chemistry will be highly variable and species-specific.

Similar results were observed by several other investigators. In this regard, plants growing in semi-arid environments have various biochemical and morphological characteristics to acclimate to stressful environments, such as drought and strong light conditions (Tanaka-Oda *et al.*, 2010). Mahmoud *et al.* (2011) reported that the total content of phenolic compounds in Iraqi *T. polium* methanolic extract was found to be equal to 100.144 mg g^{-1} of dry plant. An interested paper study the effect of altitudinal gradients on the total phenols from three plant species i.e. *N. septemcrenata*, *B. undulata* and *T. polium* collected from Saint Katherine Mountain **(**Sharaf *et al.*, 2013). Their results recorded that *N. septemcrenata* species have the maximal value (7.61 mg/g dry wt.) at elevation from 2200-2400 m and minimal value (2.34 mg/g dry wt.) at elevation from 1600-1800 m; *B. undulata* have the maximal value (5.64 mg/g. dry wt.) at elevation from 2200-2400 m and minimal value (2.54 mg/g dry wt.) at elevation from 1600-1800 m and *T. polium* have the maximal value (4.66 mg/g dry wt.) at elevation from 2200-2400 m and minimal value (3.59 mg/g dry wt.) at elevation from 2000-2200 m. Our results were in compatible with these finding.

Flavonoids have been shown to exhibit their actions on membrane permeability and by inhibition of membrane-bound enzymes such as the ATPase and phospholipase (Li *et al*., 2003), Phenols responsible for antioxidant and free radical scavenging effect of plant materials (Hasanuzzaman *et al*., 2013; Seladji *et al*., 2014). Thus, it might be concluded that phenols and flavonoids has more intimate association with survival adaptability of plants at high altitudes. This might be interpreted on the bases of subjection of higher altitudes to more stressful conditions. Minimal attention has been directed toward examining the possible effects of mild environmental stresses on phytochemical composition of plants. Pennycooke *et al.* (2005) reported that chilling stress is led to elevated total phenolic content and antioxidant capacity in petunia. Similarly, high irradiation and cold stress can lead to elevated levels of flavonoids in plants (Tattini *et al*., 2005).

Regarding total sponins, total alkaloids and total tannins, the results showed that there is very narrow variation in the amounts of these secondary metabolites among the different elevation ranks for all three studied species (Table 4). Low quantity of such phytochemicals was observed to be little influence with elevation ranks under normal growth conditions. Our results were in contrast to Khaleefa *et al.* (2015) who observed that alkaloid and tannin content increases with increase in altitude. Another study, also, suggest the existence of a correlation between envi-

ronmental factors such as average annual temperature, climate, vegetation, geomorphology, altitude and tannin production **(**Mossi *et al*., 2009**)**. Tannins are considered the most important antioxidants against free radicals generated by various types of stress prevailing at higher altitudes (Ricarda *et al*., 1991). Thus these climatic changes across altitude could affect the chemical composition and ultimately the survival of some medicinal plants in high altitude regions as the stress particularly the temperature stress can affect secondary metabolites and other compounds that plants produce, which usually are the basis of their medicinal activity (Salick *et al*., 2009).

To our knowledge, there are little studies available, so far, that have investigated the effect of altitudinal gradients on total flavonoids, total phenolic acids, total saponins, total alkaloids and total tannins on desert plants in mountain ecosystem in Egypt. Actually, the increase in elevation is associated with water stress (Korner, 1999). Water stress can stimulate the accumulation of phenolic compounds (Pedrol, 2000). These responses have been correlated with an increase of tolerance against stress (Einhellig, 1996) as an adaptive role of phenolics. Thus, we can concluded that assessing of plant phytochemical contents at varying altitudes reflect the best suited altitude for commercial cultivation of the species as these phytochemicals are considered as the basis for their medicinal activity.

2. Molecular analysis using RAPD and ISSR markers

2.1. Polymorphism revealed by different RAPD and ISSR primers

In this study, RAPD and ISSR markers were successfully applied to assess the genetic diversity of the three medicinal plant species belong to family *Labiatae* (*Nepeta septemcrenata*, *Ballota undulata* and *Teucrium polium*) in Saint Katherine Mountain at the three different altitudes (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l.) under natural conditions. PCR amplification with RAPD primers led to reproducible fragment patterns for all of the evaluated species. The majority of those RAPD fragments ranged from 200 to 1000 bp (Fig. 2). The total number of amplified fragments that were generated per primer ranged from 5 to 8 and the number of polymorphic fragments ranged from 4 to 8. All the RAPD primers except for OPC-02 primer presented the highest percentage of polymorphism (100%; Table 4). It has been reported that the GC content may be a factor that determines the efficiency of a primer (Solouki *et al*., 2007) because GC content is associated with annealing temperature and is related to the generation of more DNA fragments.

The majority of ISSR fragments ranged from 200 to 1890 bp (Fig. 3). Polymorphic banding patterns were obtained with ISSR primers ranged from 6 to 11 per primer and the total number of amplified fragments ranged from 8 to 12. The oligonucleotide HB9 amplified the highest number of ISSR loci (12 bands) and gave the highest percentage of polymorphism

(92%; Table 4). The ISSR marker efficiency has been attributed to motif sequences, as well as the sequence of its anchor. The CT motif sequences produce higher polymorphism than the AT replicates **(**Hu *et al*., 2003). This suggests that the ISSR markers can be a highly informative, fast, and reliable system for the genetic diversity studies as reported by Liu *et al.* (2011).

Each of the five RAPD primers and the five ISSR primers used for analysis of the three medicinal plant species at the three different altitudes amplified different number of fragments (Table 5). Each of the ten primers yielded from 5 to 12 DNA fragments whose molecular size ranged from approximately 200 to 1890 bp. The total number of amplified was 76 bands with an average of 7.6 fragments/primer and the total number of polymorphic fragments was 64, thus, representing a level of polymorphism of 84%. The highest number of amplified fragments (60) after using all the primers was detected in *N. septemcrenata* at 2200 m a.s.l with an average of 6 fragments per primer while the lowest number (35) was detected in *T. polium* at 1800 m a.s.l with an average of 3.5 fragments per primer (Table 5). It is known that the mating system and mode of reproduction affect significantly the extent and distribution of the genetic diversity. In self-compatible species, increased homozygosity results in low levels of genetic variation, whereas species with predominantly outcrossing mating systems exhibit higher levels of genetic variation (Liu *et al*., 2011).

2.2. Phylogenetic relationship based on amplified RAPD and ISSR fragments

The similarity coefficient values among the three medicinal plant species at the three different altitudes based on band polymorphisms generated by RAPD and ISSR after using the primers are presented in Table (6). The highest similarity value (0.897) was found between *B. undulate* at 1800 m a.s.l and at 2200 m a.s.l while the lowest value (0.420) was found between *B. undulate* at 1800 m a.s.l and *T. polium* at 2200 m a.s.l. The dendrogram of genetic distances among the three medicinal plant species at the three different altitudes based on band polymorphisms generated by RAPD and ISSR after using the primers is shown in (Fig. 4). The dendrogram separated the three medicinal plant species at the three different altitudes into two clusters. First cluster divided into two sub-clusters, first sub-cluster included *B. undulate* at 1800 m a.s.l and at 2200 m a.s.l added to *N. septemcrenata* at 2600 m a.s.l and *T. polium* at 1800 m a.s.l. Second subcluster included *B. undulate* and *T. polium* at 2600 m a.s.l added to *N. septemcrenata* at 1800 m a.s.l. Second cluster included *N. septemcrenata* and *T. polium* at 2200 m a.s.l.

The breeding system and altitude of origin of the species are very important in determining the differences between populations from different geographic locations (Jordano and Godoy, 2000; Rao and Hodgkin, 2002). Rodríguez-Bernal *et al.* (2013) estimated the genetic diversity among seven cosmos species based on

RAPD and ISSR markers. They found that dendrograms that were obtained with both markers were notably similar, revealing two clusters and indicating a clear genetic differentiation among the Cosmos species that were assessed. Besides this, the Cosmos species were clustered according to their collection sites. Sharma *et al*. (2015) assess the genetic diversity at molecular level and develop molecular marker for identification medicinal plants distributed in Himalayan region from 400 m to 3000 m amsl altitude range. They found fairly rich genetic diversity through RAPD marker analysis. The dendrogram of samples showed three major clusters and the samples of similar altitudes were found to be present in one cluster.

The similarity coefficient values among the three medicinal plant species based on band polymorphisms generated by RAPD and ISSR after using the primers are presented in Table (7) and the dendrogram of genetic distances is shown in (Fig. 5). The highest similarity value (0.641) was found between *B. undulate* and *T. polium* but the lowest value (0.594) was found between *B. undulate* and *N. septemcrenata* while the similarity value between *N. septemcrenata* and *T. polium* was 0.628. The dendrogram separated the three medicinal plant species into two clusters. First cluster included *B. undulate* and *T. polium* while second cluster included *N. septemcrenata*. Species belonging to family of *Labiatae* have an outcrossing mating system and can be reproduced by seeds. Therefore, the mating system and mode of reproduction could explain the high genetic diversity values that were reported in previous studies of wild species, such as *Liparis* (Chung *et al.*, 2007) and *Tadehagi* (Liu *et al*., 2011), wherein a very high level of genetic diversity has also been reported. It is known that low genetic distances among the populations indicate a close genetic relationship, whereas the genetic relationship is more distant in populations with higher genetic distances. One factor that enhances the gene exchange between individuals of geographically separated populations is the wide spreading of seeds and pollen (Byrne *et al*., 2008).

The similarity coefficient values among the three different altitudes based on band polymorphisms generated by RAPD and ISSR after using the primers are presented in Table (8) and the dendrogram of genetic distances is shown in (Fig. 6). The highest similarity value (0.632) was found between 1800 m a.s.l and 2200 m a.s.l but the lowest value (0.603) was found between 1800 m a.s.l and 2600 m a.s.l while the similarity value between 2200 m a.s.l and 2600 m a.s.l was 0.620. The dendrogram separated the three different altitudes into two clusters. First cluster included 1800 m a.s.l and 2200 m a.s.l while second cluster included 2600 m a.s.l.

Hamrick and Godt (1989) reported a strong correlation between geographical range and genetic diversity. This indicates that populations may differ with respect to all aspects of diversity and show variation in the number of alleles, the identity of

those alleles, and the effect they have on the characteristics in the population. In fact, different geographic locations nearly always differ with respect to some potentially significant ecological characteristic such as latitude, altitude, temperature and moisture availability (Rao and Hodgkin, 2002).

SUMMARY

The present investigation was carried out to study the effect of altitudinal gradients on genetic and phytochemicals contents of three medicinal plant species belong to family *Labiatae* (*Nepeta septemcrenata*, *Ballota undulata* and *Teucrium polium*) in Saint Katherine Mountain under natural conditions. All analyses were carried out through three different altitudes viz., 1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l. for the three species. Phytochemicals such as phenols, tannins, alkaloids, flavonoids and saponins were present in the methanolic extracts of aerial parts of three studied plant species but their quantity varied significantly across the different altitudes. The different species under study showed different values of total flavonoids, total phenolic acids, total saponins, total alkaloids and total tannins under the same environmental conditions. Meanwhile the same species exhibited different values of these metabolites under different elevation ranks. In general, total flavonoids and total phenolic acids were strongly increased with the increase of elevation from 1800 m a.s.l. until 2600 m a.s.l., however total saponins, total alkaloids and total tannins were slightly changed. The results are encouraging but scientific validation is necessary before being put into practice. RAPD and ISSR markers were successfully applied to assess the genetic diversity of the three medicinal plant species at the three different altitudes under natural conditions. Each of the five RAPD primers and the five ISSR primers used for analysis amplified different number of fragments. Each of the ten primers yielded from 5 to 12 DNA fragments whose molecular size ranged from approximately 200 to 1890 bp. The total number of amplified was 76 bands with an average of 7.6 fragments / primer and the total number of polymorphic fragments was 64, thus, representing a level of polymorphism of 84%. The highest number of amplified fragments (60) after using all the primers was detected in *N. septemcrenata* at 2200 m a.s.l with an average of 6 fragments per primer while the lowest number (35) was detected in *T. polium* at 1800 m a.s.l with an average of 3.5 fragments/primer. The highest similarity value (0.897) was found between *B. undulate* at 1800 m a.s.l and at 2200 m a.s.l while the lowest value (0.420) was found between *B. undulate* at 1800 m a.s.l and *T. polium* at 2200 m a.s.l. The dendrogram separated the three medicinal plant species into two clusters. First cluster included *B. undulate* and *T. polium* while second cluster included *N. septemcrenata*. The dendrogram separated the three different altitudes into two clusters. First cluster included 1800 m a.s.l and 2200 m a.s.l while second cluster included 2600 m a.s.l. Genetic polymor-

phism, the qualitative and quantitative phytochemical among the species are related to an altitudinal gradient. Assessing of genetic and phytochemical content of plants at varying altitudes can help to select elite genotype and reflect the best suited altitude for commercial cultivation of the species as these phytochemicals are considered as the basis for their medicinal activity.

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Table (1): GPS location points of plant sample sites.

Altitude	Latitude	Longitude
1800 m a.s.l	28.544798°	33.955062°
2200 m a.s.l	28.522694°	33.960365°
2600 m a.s.l	28.513188°	33.954820°

Table (2): The operon code and sequence of the primers used in the study for RAPD and ISSR procedure.

Code	Sequence (5′- to 3′-)	Code	Sequence (5′- to 3′-)
OPA-05	AGG GGT CTT G	HB9	(GT)6GG
OPB-10	CTG CTG GGA C	HB11	(GT)6CC
OPB-18	CCA CAG CAG T	HB13	(GAG)3GC
OPC-02	GTG AGG CGT C	HB14	(CTC)3GC
OPD-07	TTG GCA CGG G	HB15	(GTG)3GC

Table (3): Preliminary phytochemical screening of *N. septemcrenata*, *B. undulata* and T. *polium* plants collected from three different altitudes [1800, 2200 and 2600 m above sea level (a.s.l.)] of Saint Katherine Mountain. T: denotes trace amount.

Group	Test	Methanolic Extracts								
		N. septemcrenata			*B. undulata*			*T. polium*		
		1800 m a.s.l	2200 m a.s.l	2600 m a.s.l	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l
Alkaloids	Wagner's test	+ve	+ve	+ve	+ve	+ve	+ve	T	T	T
	Dragndorrf test	+ve	+ve	+ve	+ve	+ve	+ve	T	T	T
Glycosides	Glycosides test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	Modified borntrager's test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Cardiac glycosides	Legal's test	-ve	-ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
Saponins	Foam test	T	T	T	T	T	T	+ve	+ve	+ve
	Blood hemolysis test	T	T	T	T	T	T	+ve	+ve	+ve
Phenol	Ferric chloride test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	-ve	-ve
Sterol	Salkawskis test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	Libermann burchard's test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Tannins	Lead acetate test	+ve	T	T	+ve	T	T	T	T	T
	Gelatin test	+ve	T	T	+ve	T	T	T	T	T
Flavonoids	Shinoda's test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
	NaOH test	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve	+ve
Diterpene	Xanthoproteic test	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Volatile oil	Stem distillation	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Table (4): Phytochemical variations of three study plants (*N. septemcrenata*, *B. undulata* and *T. polium* collected from three different altitudes [1800, 2200 and 2600 m above sea level (a.s.l.)] of Saint Katherine Mountain. Each value is mean of 3 replicates. ± standard error of means.

Total active materials	*N. septemcrenata*			*B. undulate*			*T. polium*		
	2600 m a.s.l	2200 m a.s.l	1800 m a.s.l	2600 m a.s.l	2200 m a.s.l	1800 m a.s.l	2600 m a.s.l	2200 m a.s.l	1800 m a.s.l
Total flavonoids (mg/gm rutin)	145 ± 0.21	189 ± 0.20	201 ± 0.11	168 ± 0.40	190 ± 0.10	208 ± 0.40	195 ± 0.30	226 ± 0.20	257 ± 0.30
Total phenolic acids (mg/gm Gallic acid)	235 ± 0.40	245 ± 0.30	270 ± 0.30	211 ± 0.20	240 ± 0.40	267 ± 0.10	254 ± 0.60	280 ± 0.30	299 ± 0.10
Total Saponins (%)	0.9 ± 0.20	0.81 ± 0.20	0.87 ± 0.20	0.7 ± 0.10	0.8 ± 0.10	0.72 ± 0.30	0.4 ± 0.20	0.6 ± 0.10	0.51 ± 0.30
Total Alkaloids (%)	1.4 ± 0.3	1.4 ± 0.40	1.3 ± 0.12	1.4 ± 0.20	1.5 ± 0.10	1.5 ± 0.10	1.2 ± 0.50	1.31 ± 0.40	1.1 ± 0.23
Total Tannins (%)	2.1 ± 0.20	2.1 ± 0.50	2.1 ± 0.40	1.4 ± 0.40	1.45 ± 0.20	1.3 ± 0.30	0.7 ± 0.20	0.9 ± 0.50	0.8 ± 0.30

Table (5): Total number of fragments, polymorphic fragments and percentage of polymorphism obtained per each RAPD and ISSR primer for the three medicinal plant species at the three different altitudes.

Primers	Range of fragment sizes (bp)	*N. septemcrenata*			*B. undulate*			*T. polium*			Total No. of fragments	Polymorphic fragments	Polymorphism %
		1800 m a.s.l	2200 m a.s.l	2600 m a.s.l	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l			
RAPD													
OPA-05	230-820	2	2	3	4	4	5	1	3	5	7	7	100
OPB-10	230-930	4	6	4	3	2	2	2	6	4	8	8	100
OPB-18	250-1000	4	3	4	3	2	2	3	2	5	5	5	100
OPC-02	200-1000	3	2	4	2	4	2	2	4	4	5	4	80
OPD-07	300-900	5	5	4	3	3	3	3	5	2	5	5	100
ISSR													
HB9	420-1890	3	12	12	12	12	2	12	1	7	12	11	92
HB11	240-1330	2	8	3	3	2	2	2	8	5	8	6	75
HB13	270-1570	3	9	4	4	4	4	4	8	2	9	7	78
HB14	300-1800	7	8	2	7	7	7	3	1	7	8	7	88
HB15	200-1600	9	7	7	6	1	9	3	9	9	9	6	**67**
Total		42	**60**	47	46	41	38	**35**	47	50	76	64	84
Average		4.2	**6.0**	4.7	4.6	4.1	3.8	**3.5**	4.7	5.0	7.6	6.4	

Table (6): The similarity coefficient values among the three medicinal plant species at the three different altitudes based on band polymorphisms generated by RAPD and ISSR after using the primers.

Species and altitudes		*N. septemcrenata*			*B. undulate*			*T. polium*		
		1800 m a.s.l	2200 m a.s.l	2600 m a.s.l	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l
N. septemcrenata	1800 m a.s.l	1.000								
	2200 m a.s.l	0.538	1.000							
	2600 m a.s.l	0.603	0.628	1.000						
B. undulate	1800 m a.s.l	0.628	0.654	0.846	1.000					
	2200 m a.s.l	0.603	0.603	0.795	***0.897***	1.000				
	2600 m a.s.l	0.756	0.526	0.615	0.692	0.692	1.000			
T. polium	1800 m a.s.l	0.551	0.603	0.821	0.744	0.795	0.692	1.000		
	2200 m a.s.l	0.615	0.590	0.474	***0.420***	0.423	0.654	0.449	1.000	
	2600 m a.s.l	0.718	0.564	0.628	0.731	0.628	0.705	0.551	0.564	1.000

Table (7): The similarity coefficient values among the three medicinal plant species based on band polymorphisms generated by RAPD and ISSR after using the primers.

Species	*N. septemcrenata*	*B. undulate*	*T. polium*
N. septemcrenata	1.000		
B. undulate	0.594	1.000	
T. polium	0.628	0.641	1.000

Table (8): The similarity coefficient values among the three different altitudes based on band polymorphisms generated by RAPD and ISSR after using the primers.

altitudes	1800 m a.s.l	2200 m a.s.l	2600 m a.s.l
1800 m a.s.l	1.000		
2200 m a.s.l	0.632	1.000	
2600 m a.s.l	0.603	0.620	1.000

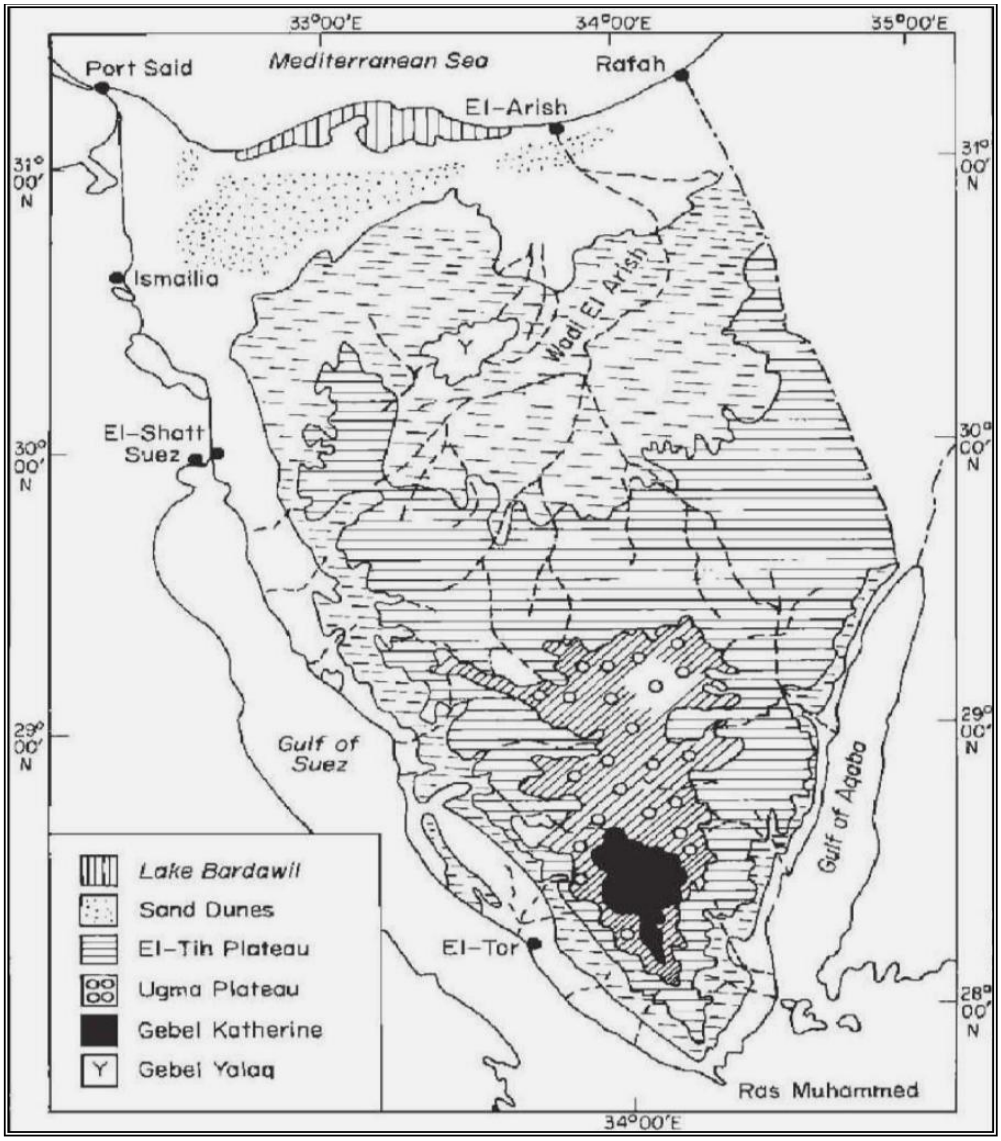


Fig. (1)**:** The main geographical features of the Sinai Peninsula. Wadis are shown by broken lines; increased elevation by heavier shading.

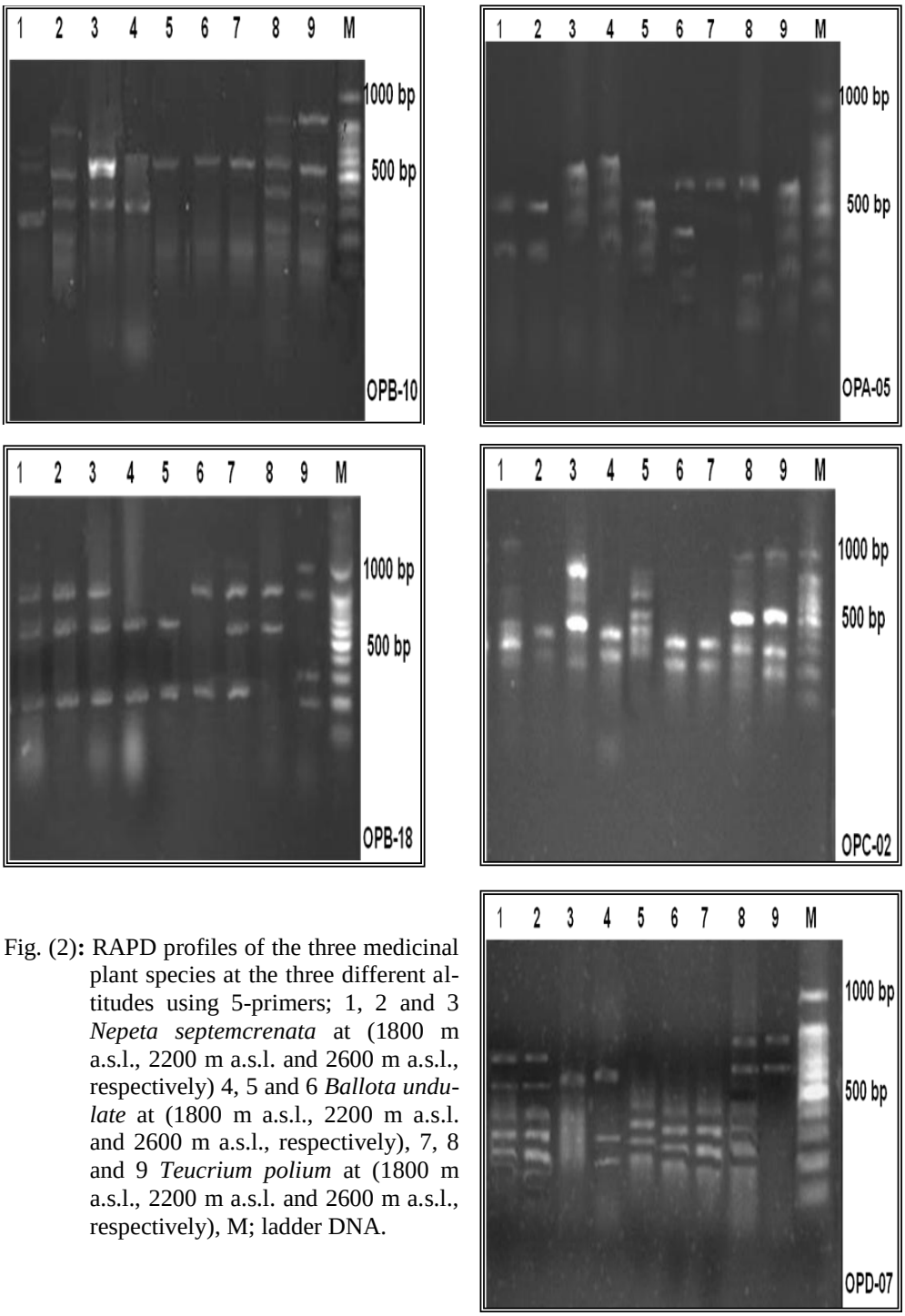
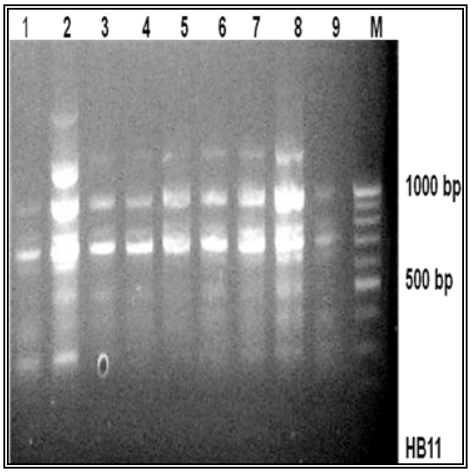
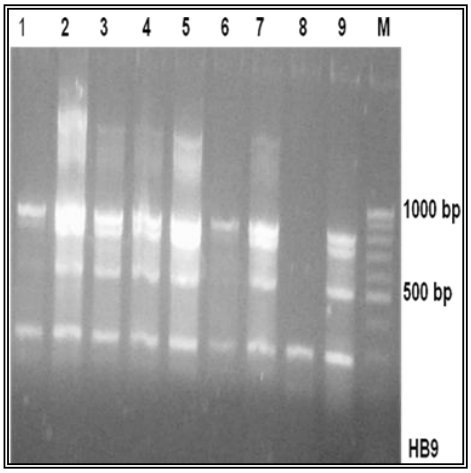
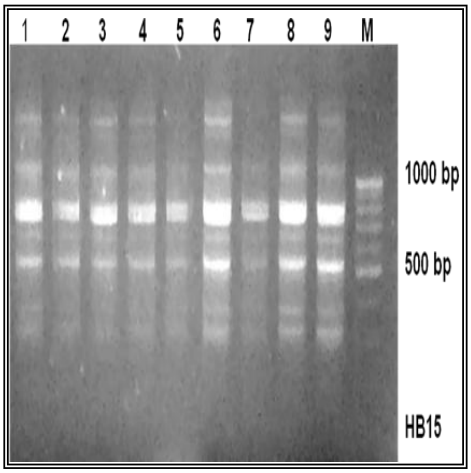
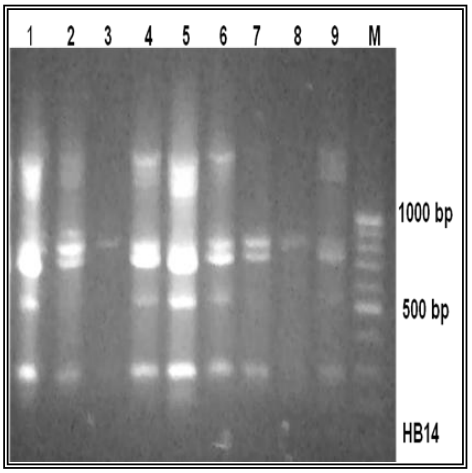


Fig. (2)**:** RAPD profiles of the three medicinal plant species at the three different altitudes using 5-primers; 1, 2 and 3 *Nepeta septemcrenata* at (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l., respectively) 4, 5 and 6 *Ballota undulate* at (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l., respectively), 7, 8 and 9 *Teucrium polium* at (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l., respectively), M; ladder DNA.









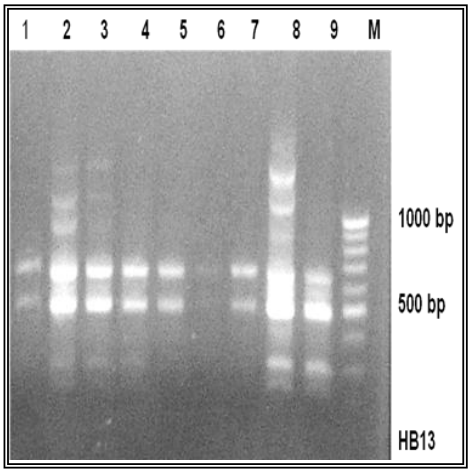


Fig. (3)**:** ISSR profiles of the three medicinal plant species at the three different altitudes using 5-primers; 1, 2 and 3 *Nepeta septemcrenata* at (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l., respectively) 4, 5 and 6 *Ballota undulate* at (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l., respectively), 7, 8 and 9 *Teucrium polium* at (1800 m a.s.l., 2200 m a.s.l. and 2600 m a.s.l., respectively), M; ladder DNA.

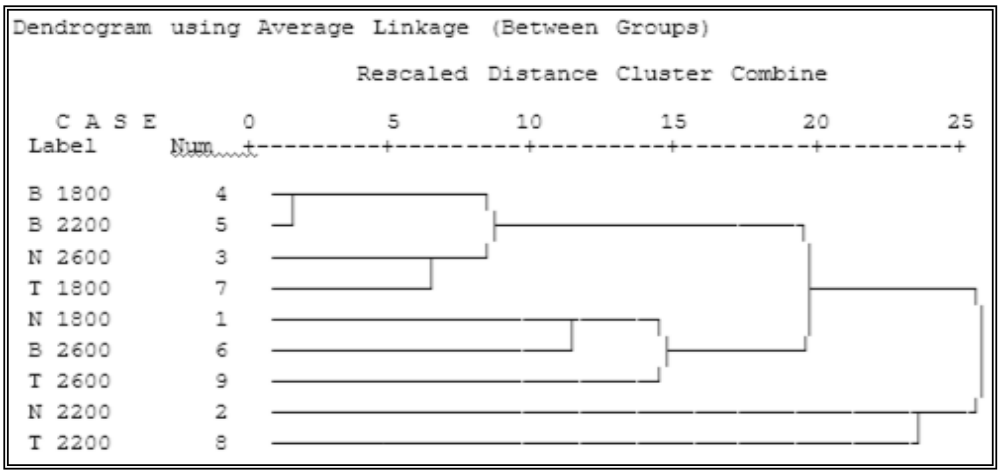


Fig. (4)**:** The dendrogram of genetic distances among the three medicinal plant species based on band polymorphisms generated by RAPD and ISSR after using the primers.

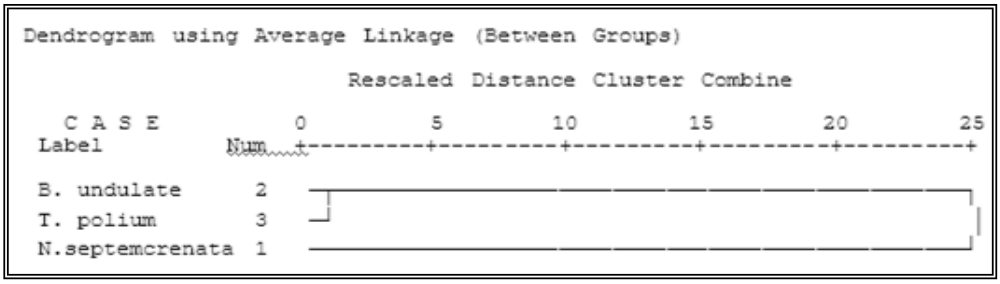


Fig. (5): The dendrogram of genetic distances among the three medicinal plant species based on band polymorphisms generated by RAPD and ISSR after using the primers.

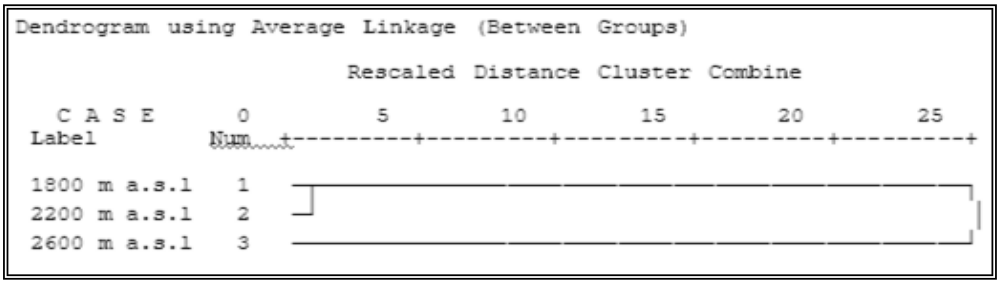


Fig. (6): The dendrogram of genetic distances among the three different altitudes based on band polymorphisms generated by RAPD and ISSR after using the primers.